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# Combining Ultrasound-Assisted Extraction and a Microliter Colorimetric Assay for the Streamlined Determination of Urea in Animal Feedstuff

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**ABSTRACT:** This paper proposes a novel analytical strategy for the routine determination of urea in animal feedstuffs, combining an ultrasound-assisted miniaturized extraction protocol with a microplate colorimetric assay based on the reaction between 4-dimethylaminobenzaldehyde and urea. In order to accelerate the extraction, we introduced an ultrasound-assisted miniaturized protocol and compared it with both classic and miniaturized alternatives. The potential interference of amino acids was bypassed by shifting the detection wavelength from 435 to 450 nm. Urea could be quantified in the range 0.05−1.00% (w/w) with high precision (RSD < 5%). The results were in agreement with a commercial enzymatic method, demonstrating the accuracy and selectivity of the assay. The miniaturization led to a 50 times downscale when compared to the official method, resulting in a reduction of at least 90% in chemical consumption per determination, contributing to a more "green" and sustainable analytical methodology.

**KEYWORDS:** *urea, ultrasound-assisted extraction, microplate assay, 4-dimethylaminobenzaldehyde (4-DMAB), animal feedstuff, green analytical chemistry*

## INTRODUCTION

Urea has been used worldwide in ruminant rations for decades,[1] being authorized in the EU as a feed additive from the beginning of rumination.[2,3] Along with the natural occurrence of nonprotein nitrogen in many common feedstuffs, urea can be added to the diet as a replacement for a part of the protein. Its use is closely linked to its cost in relation to high-protein feeds. In the rumen, urea is hydrolyzed to ammonia and carbon dioxide by the bacterial urease. Ammonia is then used by the ruminal microflora for synthesis of microbial proteins, which constitute the main amino acids (AA) source for the animals. The surplus ammonia is absorbed through the rumen wall into blood and detoxified to endogenous urea in the liver. Nevertheless, when the production of ammonia exceeds its utilization by ruminal microbes and the ability of liver to metabolize it, toxicity can occur.[4,5] For this reason, monitoring urea content in ruminant feedstuff is essential to ensure the quality of the product and minimize toxicological risks during animal production.

Urea can be determined by the direct analysis of the molecule or by its reaction products (after derivatization or enzymatic degradation).[6] The most recent approaches for urea determination have benefited from the latest evolutions in the analytical field, such as liquid chromatography,[7] molecularly imprinted polymers,[8] biosensors,[9] or microfluidics,[10−12] mainly being applied in clinical diagnostics,[6] environmental monitoring,[13] and food quality control.[14,15]

Conversely, the quantification in animal feedstuff matrices still relies on the official methods,[16,17] based on the colorimetric reaction between 4-dimethylaminobenzaldehyde (4-DMAB) and urea,[18,19] without any significant advance over the last 3 decades.[20] Moreover, the classic sample preparation protocol implies an extraction of the sample during 30 min using 500 mL of solvent. These methodologies are labor intensive, require high amounts of chemicals, and produce a significant volume of effluent, characteristics that are incompatible not only with routine quality control protocols but also with current "green" analytical chemistry requirements.[21,22]

In this context, miniaturization and accelerated sample preparation may drive new advances in this methodology toward high-throughput, low-cost, and sustainability. Miniaturization already contributed to the development of microplate assays for monitoring urea in growing media of cell cultures[23] and soil extracts.[13] However, these assays are based on slow reactions at high temperatures and, thus, are untailored for a reduced analysis time. Regarding the sample preparation, the use of ultrasound radiation can contribute for its abbreviation.

Ultrasound radiation has been applied in different steps of the analytical workflow,[24,25] but with a particular emphasis on sample preparation.[26,27] Cavitation phenomena originated by ultrasound waves make it an effective tool for a diversity of sample treatment operations, such as digestion,[28] extraction,[29] or chemical derivatization.[30] Considering solid samples, ultrasound can contribute to accelerated digestion, slurry formation, or analyte leaching.[31] The latter case fits our

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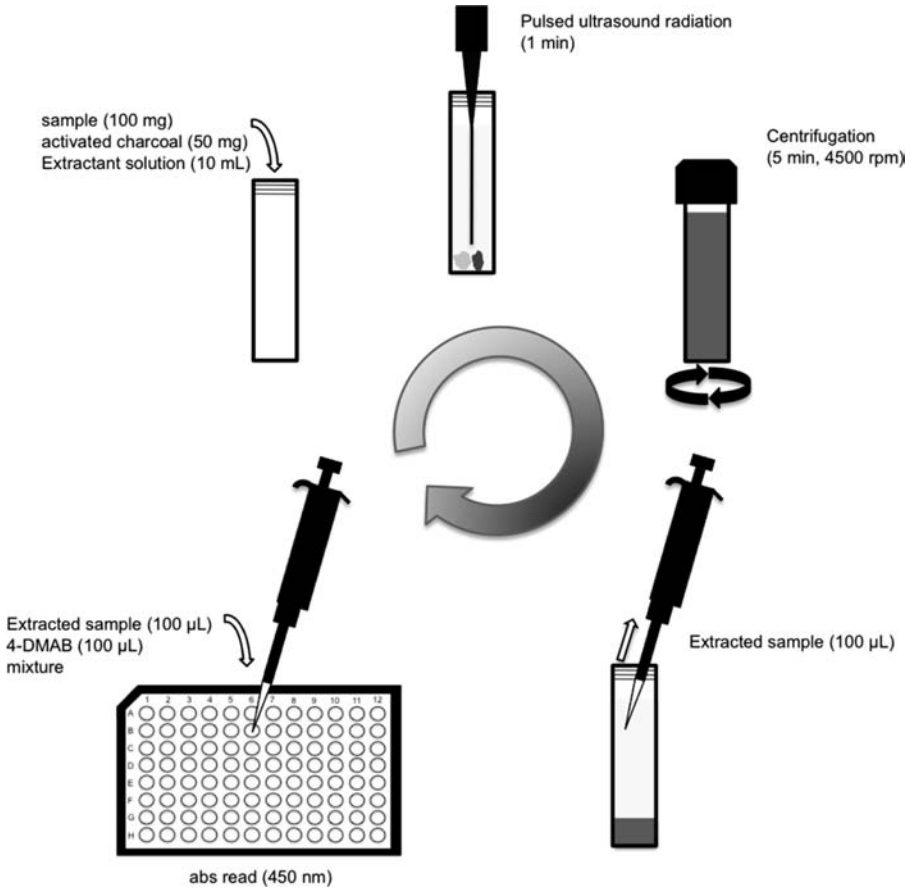


**Figure 1.** Schematic representation of the analytical workflow adopted for the determination of urea in animal feedstuff samples.

particular purposes, where the fine particles of the sample can be effectively dispersed in the extractor solvent, contributing to a fast analyte leaching.

On the basis of this background, we aimed a streamlined and environmentally friendly method for the determination of urea in feedstuff samples, combining an accelerated sample preparation protocol based on ultrasound-assisted extraction with the reaction between 4-DMAB and urea under microplate format. Furthermore, we investigated the potential interference from free AA in the dye formation, which have been recently identified as a potential source of inaccuracy for this determination.[32]

## EXPERIMENTAL SECTION

**Reagents and Solutions.** All aqueous solutions were prepared with ultrapure water (maximum conductivity of 0.055 $\mu$S cm$^{-1}$) produced by a Sartorius water purification system (arium pro, Goettingen, Germany). A 400 mmol L$^{-1}$ solution of 4-dimethylaminobenzaldehyde (4-DMAB) (Merck, Darmstad, Germany) was prepared by the dissolution of the appropriate amount of reagent in 10.0 mL of ethanol (96%, v/v) (Panreac, Barcelona, Spain) followed by the addition of 1.00 mL of 8.0 mol L$^{-1}$ hydrochloric acid, which was previously diluted from a 37% (w/w) commercial solution (Panreac). Urea (Sigma-Aldrich, St. Louis, MO) stock solution (1000 mg L$^{-1}$) was prepared by weighing 100.00 mg of solid reagent and dissolving it in 100.0 mL of ultrapure water. Working standards were prepared in the microplate wells by stepwise dilution of an intermediate 100.00 mg L$^{-1}$ solution.

The potential interference of the AA in the reaction between 4-DMAB and urea was tested using AA solutions obtained from dilution of a commercial standard (Sigma-Aldrich) that included 18 different compounds with a individual concentration of 2.50 $\mu$mol mL$^{-1}$ (excepting L-cystine, with a concentration of 1.25 $\mu$mol mL$^{-1}$).

For extraction of feedstuff samples, carrez I [zinc acetate (Panreac) and acetic acid (Sigma-Aldrich)] and carrez II [potassium hexacyanoferrate (II) (Panreac)] were prepared according to the official method.[17] Both solutions were diluted 50 times before addition to the extraction tube, where 100 mg of activated charcoal (Sigma-Aldrich) has been previously added.

**Equipment.** Samples, extraction solvent, and activated charcoal were homogenized with the assistance of a Branson sonifier (model SLPe, Danbury, CT) equipped with a 1/8″ microtip probe using the following settings: amplitude, 50%; pulsed mode, emission during 10 s followed by a pause of 5 s; irradiation time:, 1 min). After extraction, samples were centrifuged during 5 min at 3000*g* in a benchtop centrifuge (Sigma S2-5, Sigma-Aldrich).

All determinations were performed under microplate format (96-well microplates, well volume 340 $\mu$L, Orange Scientific, Braine-l'Alleud, Belgium) using a Synergy HT reader (Bio-Tek Instruments, Winooski, VT) controlled by Gen 5 software (Bio-Tek Instruments). Absorbance measurements were conducted at 25 °C and values were recorded approximately 1 min after the insertion of the microplate into the reader. During preliminary studies of the reaction, absorbance was monitored at 420, 435, and 450 nm during 30 min (one read every 3 min). Spectra of the dyes formed between 4-DMAB and urea and/or AA were obtained with a V-660 dual-beam spectrophotometer (JASCO, Easton, MD).

**Protocol Sequence.** The analytical protocol is represented in Figure 1. Sample and activated charcoal were weighed to a 12 mL plastic tube, and 5.00 mL of each carrez solution were added. The mixture was then irradiated for 1 min and then centrifuged at 3000*g* during 5 min. Subsequently, 100 $\mu$L of the supernatant was transferred to the microplate well, where the reagent (100 $\mu$L) was added and

mixed with a multichannel pipet (8−10 aspiration/dispense cycles, 100 μL) before being read at 450 nm.

**Samples.** Feedstuff samples for ruminant feeding, comprising raw material mixtures, concentrates, and total mixed rations, were obtained from Cooperativa Agrícola de Vila do Conde, CRL (Vila do Conde, Portugal). After collection, the samples were dried during 24 h at 65 °C and ground to a particle size of 0.5 mm. For comparison purposes, the three kinds of matrices were extracted following the procedure of the official method[17] and the miniaturized method (including or excluding ultrasound radiation). For this particular study, total mixed ration samples were spiked with urea.

**Urea Reference Methodology.** A urea/ammonia kit (NZYTech, Lisbon, Portugal) was used as reference methodology for accuracy assessment. This method is based on the conversion of NADPH into $NADP^+$ (monitored at 340 nm) through the combined action of urease and glutamate dehydrogenase. The amount of $NADP^+$ formed is stoichiometrically proportional to the amount of urea and/or ammonia present in the sample. Absorbance measurements were carried out under microplate format (in the same microplate reader, using 96-well flat-bottom UV transparent microplate, well volume 370 μL, BD Falcon, San Jose, CA) after adjustment of the protocol according to the "alternative procedures" brochure provided by the manufacturer.[33] All extracts were diluted 20 times before determination.

**Statistical Analysis.** Regressions between urea content and read absorbance for each calibration curve where AA were added at constant concentrations were obtained using the PROC REG of SAS (version 9.1, SAS Institute, Inc., Cary, NC). Regression coefficients (intercepts and slopes) were compared using dummy variables through PROC REG of SAS.

To streamline the assay, a variables normality test was performed according to numerical methods (descriptive and Kolmogorov−Smirnov test). Data were analyzed using the PROC MIXED of SAS to evaluate the effect of several assay parameters, including extraction strategy (official method,[17] miniaturized protocol, and ultrasound-assisted miniaturized protocol), detection wavelength (435 or 450 nm), and absorbance reading time (1 or 30 min after microplate insertion into the reader), and all double interactions as fixed effects, feed sample as random effect, and the random residual error. As interactions between fixed effects were never significant ($P > 0.15$), they were removed from the model. When a significant effect was observed the means were compared using the least-squares difference method.

## RESULTS AND DISCUSSION

**Development of Microplate Assay for the Determination of Urea in Animal Feedstuffs.** The protocol for the determination of urea consisted of a microplate assay based on a colorimetric reaction between urea and 4-DMAB.[18] The choice of this reaction was based not only on the previous reports that successfully used it in the quantification of urea in our target matrices[16,17,20,34] but also in the possibility to operate at room temperature, which simplified and minimized potential errors in the analytical workflow. Therefore, the different physical and chemical parameters involved in the reaction between 4-DMAB and urea—concentration of reagent, amount of acid added to it, and temperature—were studied. For the different experimental conditions, we established calibration curves (up to 100.0 mg $L^{-1}$ of urea) under different reaction conditions and evaluated its influence on the sensitivity of the assay.

The dye formation between urea and 4-DMAB requires an appropriate concentration of the colorimetric reagent combined with an acidic milieu.[19] Starting on the experimental conditions previously reported for this determination,[16,17,20,34] with a concentration of approximately 100 mmol $L^{-1}$, our results showed a positive linear correlation between sensitivity and 4-DMAB concentrations from 50.0 to 500.0 mmol $L^{-1}$ {slope = $4.97 \times 10^{-6}$ ($\pm 3.77 \times 10^{-7}$) × [4-DMAB] − $7.61 \times 10^{-6}$ ($\pm 1.14 \times 10^{-4}$), $r^2 = 0.997$}. Slope and 4-DMAB concentration are expressed in UA $mg^{-1}$ L and mmol $L^{-1}$, respectively) and the concentration of 400 mmol $L^{-1}$ was selected, because it ensured an appropriate sensitivity for the determination at different detection wavelengths.

The acidity of the reaction milieu is another key aspect of this reaction due to the implications on the dye formation and the solubility of 4-DMAB in aqueous solutions.[19,35,36] Its influence was studied by adding a fixed volume of HCl (equivalent to 10% of the DMAB volume) in different concentrations to the ethanolic (96% v/v) 4-DMAB solutions. From concentrations between 8.0 and 12.0 mol $L^{-1}$, the sensitivity was kept constant (variation lower than 5%), whereas for lower concentrations (4.0−8.0 mol $L^{-1}$) it dropped (14 and 30%, respectively), until the formation of precipitate at the lowest concentration used (2.0 mol $L^{-1}$). On the basis of these observations, we opted for HCl at a concentration of 8.0 mol $L^{-1}$, corresponding to 0.36 mol $L^{-1}$ in the well, which was the minimum concentration that ensured the highest sensitivity.

The official methods[16,17,34] require temperature control for the reaction. For example, the FAO method[16] describes color development at 20 ± 4 °C during 15 min and recommends one "to carry out the measurements for establishing the calibration curve and for the test samples at the same time" due to "the strong influence of the temperature on the absorbance". In this work, the microplate reader controlled the temperature as a built-in function, which is a clear advantage of its utilization. After performing absorbance monitoring during 30 min at different temperatures [room temperature (~20 °C) to 40 °C], we observed two distinct behaviors: for temperatures up to 25 °C, the absorbance values increased (approximately 5%, at room temperature) or stayed constant (25 °C) along the absorbance monitoring; whereas for temperatures above 25 °C, the absorbance values dropped within the same time frame. Slope values of the calibration curves reflected this fact. Considering the first absorbance reading, slope was similar for all temperatures (<5% variation), while for the last reading (30 min) it dropped for temperatures above 25 °C. For example, at 35 °C, the slope decreased around 11% when compared with the value at 20 °C. Thus, a temperature of 25 °C was chosen to measure absorbance, due to its minimal influence in the reaction progress and due to the easy control by our microplate reader (able to control temperatures 3 °C above room temperature).

The mixture between reagent and sample is not trivial due to the different densities and viscosities of the ethanolic (4-DMAB) and aqueous (urea extracts) solutions that are present. Official methods[16,17,34] report a mixture in test tubes, which is not compatible with a low-waste, high-throughput microplate assay. On the other hand, the degree of mixing provided by the shaking function of the reader was inappropriate and led to impaired precision caused by incomplete reaction and/or optical artifacts. To overcome this, we applied aspiration and dispensing cycles with a multichannel micropipet to get a homogeneous solution (8−10 cycles with a volume of 100 μL). This procedure took about 10 min for a 96-well plate, performed on a column-to-column basis. To minimize potential impact in the different reaction timings associated with this procedure, we adopted a microplate layout where the urea standards were positioned at the center of the plate, sided by samples.

The sensitivity of the method can also be tailored by changing the total analysis volume, corresponding to a 1:1 proportion between reagent and sample. In our final conditions, we adopted a volume of 200 $\mu$L to facilitate the mixing procedure, though it is possible to handle a maximum of 300 $\mu$L, with an extra 50% gain in the slope value of the calibration curve as a result of the increased optical path.

**Assessment of Interferences.** Molecules containing primary amino groups, such as hydrazine and semicarbazide, are potential interferent species of the reaction between 4-DMAB and urea.[18] In food and feed samples, the main source of primary amino groups is free AA that could be part of the sample. The official methods refer to this fact[16,17,34] and suggest reading absorbance at 435 nm instead of the maximum at 420 nm to minimize potential interferences.

We investigated this effect by preparing reaction mixtures (with an urea concentration of 100 mg $L^{-1}$) with and without a mixture of free AA at two concentration levels [1000 and 2500 $\mu$mol $L^{-1}$ (N equivalent)] followed by the spectra read against a reagent blank (Figure 2). Indeed, there was a strong

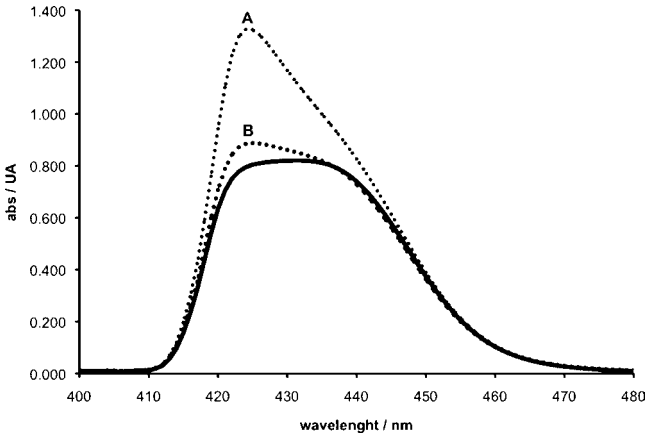


**Figure 2.** Absorption spectra of the dye formed by the reaction between 4-DMAB and urea in the absence (solid line) or in the presence (dotted line) of free amino acids in a concentration of 2500 $\mu$mol $L^{-1}$ (A) and 1000 $\mu$mol $L^{-1}$ (B), expressed as N equivalent.

absorbance from AA at 420 nm that dropped at 435 nm. For example, at 420 nm, for a concentration of 2500 $\mu$mol $L^{-1}$ (N equivalent) of AA, its contribution of the total absorbance value was 105% of the original value from urea, while at 435 nm this value represented 28%. When the concentration decreased to 1000 $\mu$mol $L^{-1}$ (N equivalent), these values were 20 and 7%, respectively. These results showed that the wavelength shift to 435 nm was insufficient to eliminate the interference. Pibarot and Pilard[32] recently reported this problem when applying the official EU method (based on the 4-DMAB reaction)[37] to the analysis of urea in pet food samples. They observed the existence of 4-DMAB−AA derivatives that resulted in a systematic overestimation of the urea content, demonstrated after submitting the same sample set to three different analytical methods (colorimetric, enzymatic, and LC−MS).

We circumvent this problem by shifting the detection wavelength from 435 to 450 nm. At 450 nm, the contribution of AA to the total absorbance value (Figure 2) was 8 and 3%, for AA concentrations of 2500 and 1000 $\mu$mol $L^{-1}$ (N equivalent), respectively. To complement these results, a series of calibration curves, where AA were added at constant concentrations [up to 2500 $\mu$mol $L^{-1}$ (N equivalent)], were established (Figure 3). For both wavelengths, sensitivity remained constant in the whole range of AA concentrations added and the intercept value had a similar behavior up to 500 $\mu$mol $L^{-1}$ (N equivalent). Nevertheless, for higher AA concentrations, intercept values increased when the reaction product was detected at 435 nm and kept constant at 450 nm, demonstrating that at this latter wavelength the contribution of AA to the final absorbance value can be neglected. This is confirmed by the statistical analysis of the results, where intercepts from the calibration curves at 435 nm were significantly different ($P < 0.001$), whereas at 450 nm neither intercepts nor slopes of calibration curves were significantly different ($P > 0.05$).

**Ultrasound-Assisted Extraction of Animal Feedstuff Samples.** In order to streamline the assay, we designed an ultrasound-assisted extraction protocol that assured a repeatable dispersion of sample and activated charcoal into the extractor solvent. Therefore, our experimental setup consisted of the extraction of 11 samples (five raw material mixtures, three concentrates, and three total mixed rations)—representing the complete framework of application—following three different protocols: official method[17] (A), miniaturized protocol (B), and ultrasound-assisted miniaturized protocol (C). Ultrasound settings were adjusted by looking for a negligible heating of the mixture. After extraction, all samples were centrifuged and the supernatants analyzed using the microplate assay. Effects of extraction strategy, detection wavelength, and absorbance reading time on sample urea content are presented in Table 1.

The extraction strategy significantly affected the analytical results ($P < 0.001$). The official method (A) and the ultrasound-assisted miniaturized extraction (C) gathered similar results, and the miniaturized extraction led to lower urea concentrations. The detection wavelength (435 and 450 nm) and the absorbance reading time (1 and 30 min after plate insertion) did not significantly affect the determination.

Considering that urea was present in free form and is largely soluble in water, we hypothesize that ultrasound promoted a uniform dispersion of the particles into the extractor solvent, improving the wettability of the sample and, consequently, the extraction. This effect could also enhance the dispersion of the activated charcoal, contributing to a more efficient adsorption of matrix components that could have intrinsic absorbance at the detection wavelength. We also observed that the use of activated charcoal did not interfere with the measurements, in agreement with the report from Schramm and Aines.[38] For the total mixed ratio samples spiked with urea at 0.80 and 1.00% (w/w) and submitted to the official method and miniaturized ultrasound-assisted extraction protocols, the recovery values ranged between 85 and 107%, regardless of the detection wavelength.

**Characterization of the Method and Application to the Analysis of Feedstuff Samples.** The characterization of the new method considered its linear range, sensitivity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and accuracy. Detection was possible at 450 and 435 nm, although in the latter case the level of free AA present in the sample may affect the results.

Linearity ($r^2 > 0.999$) was found in the range 5.00−100 mg $L^{-1}$, equivalent to 0.05−1.00% (w/w) of urea in feedstuff samples for the applied extraction conditions.

The sensitivity of the assay depended of the detection wavelength. At 435 nm, the typical calibration curve {abs = 0.202 ($\pm$0.002) + 0.00457 ($\pm$0.000 03) × [urea]} showed a


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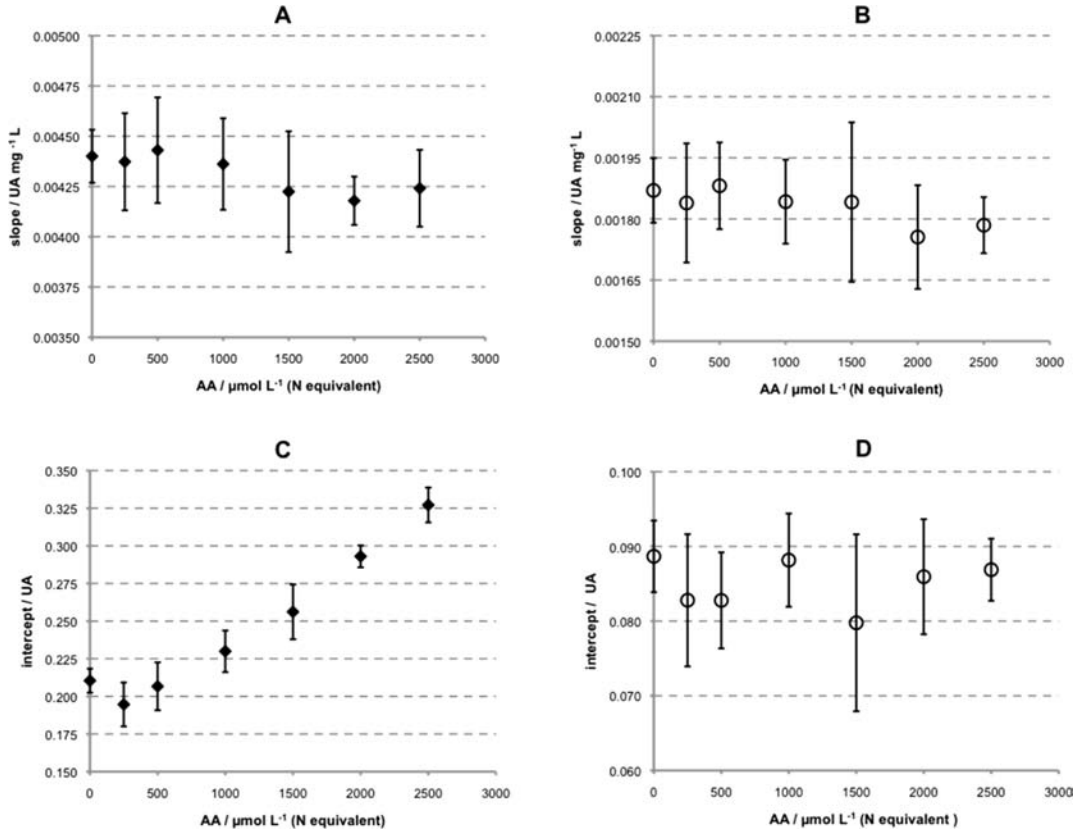


**Figure 3.** Influence of the amino acids (AA) added to urea standards (up to 100 mg $L^{-1}$) in the parameters of calibration curves (slope, A, B; intercept, C, D) obtained at 435 nm (◆) and 450 nm (○). Error bars represent the limits of the 95% confidence level intervals. $r^2 > 0.997$ and >0.994 for calibration curves at 435 and 450 nm, respectively.

**Table 1. Effects of Three Different Extraction Strategies (A, Official Method; B, Miniaturized Extraction; C, Ultrasound-Assisted Miniaturized Extraction) and Absorbance Detection Settings (Read Time after Plate Insertion and Detection Wavelength) on Urea Values Obtained[a]**

| extraction strategy | | | | | read time/min | | | | wavelength/nm | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | B | C | SEM | *P* | 1 | 30 | SEM | *P* | 435 | 450 | SEM | *P* |
| 0.491 a | 0.389 b | 0.513 a | 0.1067 | <0.001 | 0.462 | 0.467 | 0.1065 | 0.665 | 0.466 | 0.463 | 0.1065 | 0.855 |

[a]Values that share a common letter are not statistically different ($P > 0.05$). SEM: standard error of the mean.

sensitivity value approximately 2.5 times higher than the one found at 450 nm {abs = 0.088 (±0.001) + 0.00191 (±0.000 01) × [urea]}, covering the same linear range. The values in parentheses correspond to the limits of the 95% confidence level intervals, and urea concentration is expressed in mg $L^{-1}$.

For both detection wavelengths, LOD and LOQ were 1 and 5 mg $L^{-1}$, respectively, and they were calculated by considering the urea concentration equivalent to the analytical signal obtained by adding the blank signal to 3 (LOD) and 10 times (LOQ) the corresponding standard deviation.[39]

Relative standard deviation (RSD%) was used to measure the precision of the assay. Values were <2.5% and <5% ($n = 4$) for standard solutions and samples, respectively. During this development stage, we kept the number of replicates as four, in order to trace potential problems with the mixture at the microplate. Nevertheless, the analytical output showed high precision, and the number of replicates can be reduced for routine application.

To evaluate accuracy, we determined the urea concentration in 11 samples (six concentrates and five raw material mixtures) by the miniaturized methodology ($C_{min}$) and also by a commercial enzymatic kit ($C_{enz}$), with relative deviations ≤5.3% (Table 2). The values obtained by both methods exhibited a linear relationship, described by the equation $C_{min}$ = 0.00084 (±0.0251) + 0.990 (±0.042) × $C_{enz}$ ($r^2 = 0.997$, $n = 11$). The values in parentheses correspond to the limits of the 95% confidence level intervals. Hence, it is demonstrated that the values of slope and intercept are not significantly different from 1 and 0, and consequently, both methods are equivalent.[39] These results also evidenced the selectivity of the 4-DMAB microplate approach for the analysis of urea in these matrices because AA interference has not been reported for the comparative method applied.[32]

**Environmental Performance of the New Methodology.** The present work is a clear example of the potential evolution of analytical methods toward green and sustainable protocols.[22,26,40,41] Miniaturization led to a 50 times downscale of the protocol volumes when compared to the official methods.[16,17,34] The consequence was a reduction of at least 90% in chemical consumption and 98% in effluent generation per determination. For example, HCl consumption dropped from 0.488 to 0.040 mmol, and the extraction volume was

**Table 2. Concentrations (Mean Value ± Standard Deviation, $n$ = 2), Expressed in Percentage of Urea (w/w), Obtained from the Analysis of Feedstuff Samples by the Proposed Miniaturized Method ($C_{min}$) and by the Enzymatic Reference Methodology ($C_{enz}$)**

| sample | $C_{min}$ | $C_{enz}$ | relative deviation/% |
|---|---|---|---|
| concentrate 1 | 0.467 ± 0.003 | 0.476 ± 0.020 | −1.9 |
| concentrate 2 | 0.579 ± 0.006 | 0.597 ± 0.010 | −3.0 |
| concentrate 3 | 0.448 ± 0.009 | 0.450 ± 0.000 | −0.4 |
| concentrate 4 | 0.464 ± 0.018 | 0.475 ± 0.006 | −2.3 |
| concentrate 5 | 0.557 ± 0.000 | 0.550 ± 0.020 | 1.3 |
| concentrate 6 | 0.481 ± 0.014 | 0.496 ± 0.015 | −3.0 |
| mixture 1 | 0.160 ± 0.002 | 0.152 ± 0.000 | 5.3 |
| mixture 2 | 0.760 ± 0.036 | 0.735 ± 0.011 | 3.4 |
| mixture 3 | 0.312 ± 0.004 | 0.320 ± 0.000 | −2.5 |
| mixture 4 | 0.828 ± 0.005 | 0.837 ± 0.010 | −1.1 |
| mixture 5 | 0.955 ± 0.008 | 0.973 ± 0.050 | −1.8 |

reduced from 500.0 to 10.0 mL. These facts also imply economical benefits. At current prices, the cost of chemical supplies per determination decreased from 0.168 € (official method) or 0.236 € (enzymatic method in microplate format) to 0.008 € (miniaturized method), a reduction of more than 95% in both cases. Furthermore, the new alternative presents a straightforward protocol, with a reduced analysis time. Hence, extraction time decreased from 30 to 1 min with the introduction of ultrasound radiation, and filtration was replaced by centrifugation, which made possible a parallel processing of several samples (up to 16 in our particular case) and also sparing the use of disposable consumables such as filters.

We also assessed the environmental performance of the method by applying the greenness profile suggested by NEMI (National Environmental Methods Index).[22,42] Although originally designed for environmental methods, we assigned the same criteria to this method, since there was a potential hazardous impact (Figure 4). Urea is a water-soluble compound, which bypassed the use of organic solvents for the extraction and made easier the accomplishment of PBT (persistent, bioaccumulative, and toxic) and hazardous requirements. None of the chemicals used was part of the Toxic Release Inventory (TRI) list,[43] implying a green quadrant for both parameters. Regarding waste production, effluent generation was 10.1 mL per determination, significantly lower than the 50 g defined by the guidelines (green quadrant). Concerning pH, the value is conditioned by the HCl necessary for dye formation. In a single well, the concentration of acid was 0.36 mol $L^{-1}$, corresponding to a pH of 0.44 [assuming a total dissociation in the ethanol−water (1:1 v/v) solution]. This value is categorized as corrosive (<2) and the quadrant was white marked.

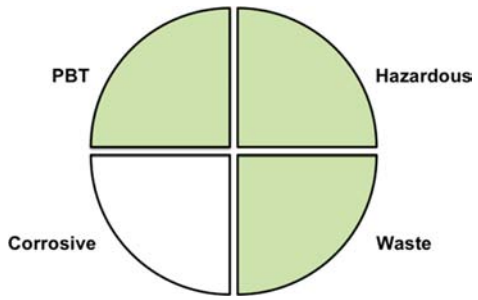


**Figure 4.** Greenness profile of the proposed methodology according to NEMI guidelines.

In conclusion, we introduced here a streamlined and environmentally friendly method for the routine determination of urea in feedstuff samples that benefited from the downscale of the reaction between 4-DMAB and urea combined with an ultrasound-assisted extraction protocol. This led to a straightforward and flexible analytical workflow and contributed to a drastic reduction in analysis time, chemical consumption, and waste generation, accomplishing green analytical chemistry demands.

This work also elucidated the role of AA as potential interference in the reaction. The detection at 450 nm surpassed this interference without jeopardizing an adequate concentration working range for real samples. This makes possible the analysis of other matrices where this potential problem can be present.

We also hope that this contribution could become a starting point for the development of new applications for feedstuff analysis that incorporate the most recent advances of analytical chemistry toward green and sustainable procedures.

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### Notes

The authors declare no competing financial interest.

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